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Application of azamulin to determine the contribution of CYP3A4/5 to drug metabolic clearance using human hepatocytes

Authors : Hugues Chanteux*, Maria Rosa*, Claude Delatour, Johan Nicolaï, Eric Gillent, Sylvie Dell'Aiera and Anna-Lena Ungell

*equally contributed to the manuscript

Affiliations

HC, MR, CD, JN, EG, SD, AL : UCB Biopharma SRL

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RUNNING TITLE

Azamulin for evaluation of $F_{mCYP3A4/5}$ in human hepatocytes

Corresponding Author : Hugues Chanteux, UCB Biopharma, Chemin du Foriest, B-1420 Braine-l'Alleud, Belgium; phone: +32(0)2.386.34.77; fax: +32(0)2.386.27.98; e-mail address: Hugues.Chanteux@UCB.com

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Abbreviations : ACN, acetonitrile; AFE, average fold error; AO, aldehyde oxidase; CYP450, cytochrome P450; DDI, drug-drug interaction; DME, drug metabolizing enzyme; DMSO, dimethylsulfoxide; $Fm_{CYP3A4/5}$, fraction metabolized by CYP3A4/5; FMO, flavin mono-oxygenase; HLM, human liver microsomes; ISEF, inter-system extrapolation factor; NCE, new chemical entity; RAF, relative activity factor; RMSE, root mean-squared error; SULT, sulfotransferase; UGT, uridine glucuronyltransferases; UPLC, ultra performance liquid chromatography

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ABSTRACT

Early determination of CYP3A4/5 contribution to the clearance of new chemical entities is critical to inform on the risk of drug-drug interactions with CYP3A inhibitors and inducers. Several in vitro approaches (recombinant P450 enzymes, correlation analysis, chemical and antibody inhibition in human liver microsomes) are available but they are usually labor intensive and/or suffer from specific limitations. In the present study, we have validated the use of azamulin as a specific CYP3A inhibitor in human hepatocytes. Azamulin (3µM) was found to significantly inhibit CYP3A4/5 (>90%) while other CYP450 enzymes were not affected (less than 20% inhibition). Since human hepatocytes were used as test system, the effect of azamulin on other key drug metabolizing enzymes (aldehyde oxidase, carboxylesterase, UGT, FMO, SULT) was also investigated. Apart from some UGTs showing minor inhibition (~20-30%), , none of these non-P450 enzymes were inhibited by azamulin. Use of CYP3A5-genotyped human hepatocyte batches in combination with CYP3cide demonstrated that azamulin (at 3µM) is inhibiting both CYP3A4 and CYP3A5 enzymes. Finally, 11 compounds with known in vivo CYP3A4/5 contribution have been evaluated in this human hepatocytes assay. Results showed that the effect of azamulin on the in vitro intrinsic clearance of these known CYP3A4/5 substrates was predictive of the in vivo CYP3A4/5 contribution. Overall, the study showed that human hepatocytes treated with azamulin provide a fast and accurate estimation of CYP3A4/5 contribution in metabolic clearance of new chemical entities.

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SIGNIFICANCE STATEMENT

Accurate estimation of CYP3A4/5 contribution in drug clearance is essential to anticipate risk of drug-drug interactions and select the appropriate candidate for clinical development. The present study validated the use of azamulin as selective CYP3A4/5 inhibitor in suspended human hepatocytes and demonstrated that this novel approach provides a direct and accurate determination of the contribution of CYP3A4/5 ($Fm_{CYP3A4/5}$) in the metabolic clearance of new chemical entities.

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INTRODUCTION

Numerous marketed drugs are metabolized by hepatic cytochrome P450 (CYP450) enzymes. Within the P450 family, CYP3A is the most abundant isozyme present in the liver and is, by far, the main CYP450 isoform involved in drug metabolism. Indeed, approximately 30% of marketed drugs are known to be substrates of CYP3A4/5 (Wienkers and Heath, 2005; Zanger and Schwab, 2013). The substrates of CYP3A4/5 are chemically diverse and this broad substrate specificity renders CYP3A4 susceptible to inhibition by a variety of drugs as demonstrated by the long list of drugs known to be CYP3A4 inhibitors (FDA, 2020). Any new chemical entity (NCE) developed nowadays and being a CYP3A4 substrate is at high risk of being prone to drug-drug interactions (DDI) in the clinic, which may significantly impact drug safety and efficacy (Desbans et al., 2014). The susceptibility of drugs towards CYP3A4 inhibition/induction is highly dependent on their so-called $F_{CL,metabolism}xF_{m,CYP3A4}$ which is the fraction of total drug clearance mediated by CYP3A4 (Zhang et al., 2007). Indeed, a NCE with $F_{CL,metabolism}xF_{m,CYP3A4} \geq 0.5$ is predicted to have an AUCR ≥ 2 when co-administered with a strong CYP3A4 inhibitor. Careful assessment of contribution of individual CYP450 isoform towards overall metabolism of a NCE is therefore crucial to accurately assess the risk as victim of DDI. Determination of the quantitative contribution of individual CYP450 enzymes to metabolism of drugs has become standard practice in the development of NCE (Zientek and Youdim, 2015; Bohnert et al., 2016; Parmentier et al., 2019). It usually involves different approaches : (a) evaluation of the inhibitory effect caused by specific CYP450 chemical inhibitors or antibodies on drug metabolism, using human liver microsomes (HLM) (Khojasteh et al., 2011) (b) metabolism by individual recombinant CYP450 enzymes scaled up with RAF/ISEF (Emoto et al., 2006) ; (c) correlation analysis comparing the rate of metabolism to activities of each CYP450 enzyme, in a set of microsomes prepared from individual donor livers (Bohnert et al., 2016). A full assessment of CYP450 contribution is labor-intensive and is usually not performed before a candidate is selected

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for clinical development. In addition, the aforementionned in vitro tests suffer from specific limitations (Ogilvie, 2008).

Given the high risk of DDI for CYP3A4 substrates, an early identification of this DDI liability is desirable to avoid any surprises once the compound is selected for further development. Typically, HLM incubated with ketoconazole are used to determine the contribution of CYP3A4 to the overall metabolism as ketoconazole has many advantages, i.e. potency, ease of use (no pre-incubation step), commercially available (Maurice et al., 1992; Zhang et al., 2002). However, ketoconazole is not as specific as it should be to be used as a CYP3A4 inhibitor in phenotyping experiments. Indeed, in HLM, at concentrations required to inhibit ~95% of CYP3A4, other CYP450 enzymes such as CYP1B1, CYP2B6, and CYP2C8/9/19 are also significantly inhibited (~20 to ~60%) (Newton et al., 1995; Moody et al., 1999; Stresser et al., 2004). Hence, this results in a potential overestimation of the contribution of CYP3A4 to overall drug clearance. In addition, HLM are not the optimal test system to measure metabolic clearance of NCE as they are lacking different key drug-metabolizing enzymes such as aldehyde oxidase (AO), xanthine oxidase, sulfotransferase (SULT) or require addition of specific uridine glucuronyltransferases (UGT) cofactor necessary to activate UGTs. Therefore, the information obtained by assays utilizing HLM should be considered carefully as it may not represent the true $F_{CL,metabolism}xF_{m,CYP3A4}$ of the NCE as the importance of other metabolic routes may have been overlooked.

Human hepatocytes are considered as gold standard test system for metabolic stability studies (Soars et al., 2007). However, their use is not common for reaction phenotyping in drug discovery even though they offer a major advantage over HLM with the presence of the full machinery of drug metabolizing enzymes which is essential to more accurately assign Fm.

The present study investigates the use of azamulin as CYP3A4/5 inhibitor (Sevrioukova, 2019) to determine the $F_{m,CYP3A4/5}$ of NCE's using cryopreserved human hepatocytes. Since selectivity is a

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key criterion to assign a $F_{m,CYP3A4/5}$ value to NCE, a huge variety of specific substrates (CYP450, UGT, SULTs, flavin monooxygenase (FMO), AO, carboxylesterase 1) were used to assess azamulin's selectivity. CYP3A4 selectivity over CYP3A5 was evaluated using human hepatocyte batches with high CYP3A5 activity treated with CYP3cide, a known specific CYP3A4 inhibitor (Walsky et al., 2012; Tseng et al., 2014). Finallly, compounds with a wide range of reported $F_{m,CYP3A4/5}$ were used to validate the assay conditions and demonstrate that this assay can be used for direct determination of $F_{CL,metabolism}xF_{m,CYP3A4/5}$ of NCE's.

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MATERIALS AND METHODS

Reagents

Cryopreserved human hepatocytes (pools of 10 or 20 donors) were obtained from Xenotech (Lenexa, KS, USA) or BioIVT (Brussels, Belgium). CYP3A5 genotyped cryopreserved human hepatocytes were obtained from BioIVT (Brussels, Belgium). Azamulin was purchased from Sigma-Aldrich (St Louis, MO, USA) and Toronto Research Chemicals (Toronto, Ontario, Canada). Midazolam was obtained from Apin chemicals (Milton, Abingdon, UK). All other compounds (CYP450 probe substrates (phenacetin, bupropion, rosiglitazone, diclofenac, (S)-(+)-Mephenytoin and dextromethorphan) and their respective metabolites (acetaminophen, hydroxybupropion, 5-hydroxyrosiglitazone, 4'-hydroxydiclofenac, 4'-hydroxymephenytoin, dextrorphan and 1'-hydroxymidazolam), phase II enzymes substrates 1-(hydroxymidazolam, 4-hydroxycoumarin, imipramine, 5-hydroxytryptophol, naloxone and S-oxazepam), aldehyde oxidase substrate (phthalazine), FMO substrates (cimetidine and ranitidine), CYP3cide (a reported specific CYP3A4 inhibitor), CYP3A4 substrates used for the validation of the assay (felodipine, simvastatin, quinidine, sildenafil, nifedipine, cerivastatin, metoprolol, loperamide, mirtazapine, zolpidem and pimozide) and various internal standards (acetaminophen-D_4, hydroxybupropion-D_6, 1'-hydroxymidazolam-D_4, 4'-hydroxydiclofenac-$^{13}C_6$, 4'-hydroxymephenytoin-D_3, dextrorphan-D_3, 5-hydroxyrosiglitazone-D4 and ketoconazole) were purchased from Sigma-Aldrich (Bornem, Belgium). CDP323 was synthetized at UCB Biopharma (Braine-l'Alleud, Belgium). Glucuronidase/arylsulfatase were obtained from Roche (Vilvoorde, Belgium). Biosolve UPLC grade (Valkenswaard, The Netherlands) water, acetonitrile, methanol, trifluoracetic acid and formic acid were used without further purification on the Microbore UPLC systems. Ammonium acetate solution at 5M and Acetic acid glacial, ≥99.85% were purchased from Sigma Aldrich (Bornem, Belgium).

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In vitro incubations to evaluate the inhibitory effect of azamulin on CYP3A4/5, in suspensions of pooled human hepatocytes, with and without pre-incubation

Cryopreserved human hepatocytes (pool of 5 males and 5 females donors from Xenotech, batch 1110211) were thawed according to the provider's information. Viability (trypan blue exclusion 0.2%) was higher than 73%. Incubations ($0.5x10^6$ cells/mL) were carried out in triplicate in 48-well plate (final volume 400 µL) placed on a rotor agitator (450 rpm), at 37°C in CO_2 environment, with WGH medium (William's medium E without phenol red, containing 2 mM of glutamine and 15 mM of Hepes). After a 5 min pre-incubation of 200 µL of suspended hepatocytes (1 million/mL) at 37°C, a second pre-incubation was initiated by the addition of 200 µL of a mix containing various concentrations of azamulin, in WGH medium. After 0, 10, 100 and 220 minutes of pre-incubation, reactions were initiated by the addition of 2 µL of midazolam at 1 mM (final concentration 5µM) and further incubated for 20 min to respect initial rate conditions of 1'hydroxymidazolam formation. Final solvent concentration in incubates was ≤ 1% v/v. All reactions were stopped by addition of two volumes of ice-cold acetonitrile (ACN). The tubes were then thoroughly mixed using a vortex mixer and centrifuged (14,000*g*, at 4°C) for 5 min. Clear supernatants were aspirated, diluted two times with water and directly analyzed by LC-MS/MS.

Determination of the inhibition potential of azamulin against CYP1A2, 2B6, 2C8, 2C9, 2C19 and 2D6 in suspensions of pooled human hepatocytes

The inhibitory effect of azamulin on various CYP450 enzymes was evaluated at 3 µM, with and without a 3 h pre-incubation, in order to determine its selectivity towards CYP3A4/5. Phenacetin O-deethylation, bupropion 4-hydroxylation, rosiglitazone 5-hydroxylation, diclofenac 4'-hydroxylation, (S)-(+)-Mephenytoin 4'-hydroxylation, dextromethorphan O-demethylation and midazolam 1'-hydroxylation were used as probe reactions of CYP1A2, 2B6, 2C8, 2C9, 2C19, 2D6

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and 3A, respectively. Probe substrates were incubated at a final concentration of 2 µM, 7 µM and 20 µM for phenacetin, bupropion and diclofenac, respectively and 5 µM for other substrates (these concentrations are at or below Km values, previously determined in our laboratory (data not shown)). Probe substrates were added to the in vitro incubates as solutions in ACN. Azamulin (0 and 3 µM, final concentration in incubates) was solubilized in H_2O/ACN (50/50, v/v). Final solvent concentration in incubates was ≤ 1%.

Viability of cryopreserved human hepatocytes (pool of 10 mixed gender donors from Xenotech, batch 1110211) was higher than 71% (trypan blue exclusion). Incubations were performed as described above. Incubation times (60 min for (S)-(+)-Mephenytoin, 20 min for midazolam and 30 min for other probe substrates) were selected in order that initial rate conditions for each CYP450 activity were respected. For rosiglitazone, before analysis, supernatants were first evaporated under N_2 and then further incubated with glucuronidase (0.358 U/mL) and arylsulfatase (0.48 U/mL) in 50 mM Na-Acetate buffer (pH 5), at 37°C for 1 hour, in order to convert glucuronide and sulfate metabolites back into 5-hydroxyrosiglitazone.

Determination of the inhibition potential of azamulin against CYP3A5 and non-CYP450 enzymes, in suspensions of pooled human hepatocytes

1'-Hydroxymidazolam (substrate of UGTs), 4-hydroxycoumarin and acetaminophen (substrates of UGTs and SULTs), phthalazine (substrate of AO), cimetidine and ranitidine (substrates of FMO), CDP323 (substrate of CES1) and several specific UGTs substrates (imipramine for UGT1A4, 5-OH tryptophol for UGT1A6, S-oxazepam for UGT2B15, propofol for UGT1A9, naloxone for UGT2B7) were incubated with suspensions of human hepatocytes (pool of 10 males and 10 females donors from BioIVT, BSU batch), in presence and in absence of azamulin (3µM). All substrates were dissolved in DMSO and azamulin in H_20/ACN (50/50, v/v). Final DMSO and ACN

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concentration in incubates were ≤ 0.01% and ≤ 1%, respectively. Cryopreserved human hepatocytes were thawed according to the provider's information. Viability was higher than 85% (trypan blue exclusion). Incubations ($1x10^6$ cells/mL) were carried out in singular, in a 48-well plate, placed on a vibrating agitator (Titramax 100, at 450rpm), in an incubator (5% CO_2:95% air, humidified atmosphere at 37°C), with WGH medium.

After a 30 min pre-incubation of 250 µL of suspended hepatocytes (2 millions/mL) at 37°C, reactions were initiated by the addition of an equivalent volume of a mix containing azamulin (or not) and the probe substrate in WGH medium. Final concentration of azamulin and probe substrate in the incubation medium were 3 µM , and 0.5 µM, respectively, except for cimetidine (2µM) and acetaminophen (10µM). At selected timepoints, samples were taken, and reactions were stopped using one volume of ice-cold ACN containing ketoconazole (0.5 µM) as internal standard (IS). Samples were subsequently centrifuged at 3,700 rpm (4°C), for 15 minutes. Clear supernatants were transferred to 96-deepwell plates, diluted two times with water and directly analyzed by LC-MS/MS or UPLC-HRMS.

To evaluate the selectivity of azamulin for CYP3A4, CYP3A5-genotyped human hepatocytes and CYP3cide (a specific CYP3A4 inhibitor) (Walsky et al., 2012) were used.

Single donor cryopreserved human hepatocytes (from BioreclamationIVT) were genotyped for CYP3A5*3 using extracted genomic DNA in a realtime PCR assay. Two different types of genotypes were used in this assay, CYP3A5*3/*3 (non-expressers of CYP3A5) and CYP3A5*1/*1 (expressing two copies of CYP3A5 and known as high expressers of CYP3A5).

Viability of hepatocytes was higher than 74% (trypan blue exclusion). CYP3cide was dissolved in H_2O/ACN (50/50, v/v). Final DMSO and ACN concentration in incubates was ≤ 0.01% and ≤1%, respectively. Incubations were performed as described in the paragraph above.

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Validation of the phenotyping assay with a set of known CYP3A4/5 substrates

Compounds (midazolam, felodipine, simvastatin, quinidine, sildenafil, nifedipine, loperamide, cerivastatin, metoprolol, mirtazapine, zolpidem, pimozide) were all dissolved in ACN. Final solvent concentration in incubates was ≤ 1%.

Incubations (substrate concentration at 0.5µM) were carried out in presence and absence of azamulin (3µM) as explained above for the substrates of non-CYP450 enzymes and detailed in Table 3.

LC-MS/MS analysis of metabolites formed from P450 probes in the in vitro samples

The metabolites of CYP450 probe substrates (acetaminophen, 4-hydroxybupropion, 4'-hydroxydiclofenac and 1'-hydroxymidazolam on one hand and 4'-hydroxymephenytoin, 5-hydroxyrosiglitazone and dextrorphan on the other hand) were quantified by separate LC-MS/MS methods (cocktail 1 and cocktail 2, respectively).

Cocktail 1 LC-MS/MS analytical method :

The qualification and description of this analytical method is available from Gerin et al (Gerin et al., 2013). Briefly, 5 volumes of sample supernatant was mixed with one volume of internal standard (IS) solution (containing acetaminophen-D_4, hydroxybupropion-D_6, 1'-hydroxymidazolam-D_4 and 4'-hydroxydiclofenac-$^{13}C_6$), evaporated to dryness under N_2 at 40°C and reconstituted in 6 volumes of water containing 0.1% trifluoracetic acid (TFA) adjusted to pH 2.4. The UPLC system used was an Agilent 1290 series instrument (Agilent technologies Santa Clara, California, USA) coupled with a Sciex API 5000 mass spectrometer (Applied Biosystems, Mississauga, Canada). The analytical column was a Zorbax Eclipse XDB-C18 (5 µm, 50.0 x 2.1 mm), operated at 40°C. Eluent A was water containing 0.1% TFA adjusted to pH 2.4 and eluent B was 100% ACN. The flow-rate was

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0.412 mL/min. A gradient elution was started at 95% A (2.16 min), then ramped successively to 75% A in 0.24 min, 60% A in 1.46 min, 50% A in 0.01 min, 20% A in 0.47 min, held at 20% A for 0.49 min, then at 10% A for 0.72 min before returning to the initial condition (95% A for 0.93 min). Data acquisition was performed and the analytical parameters were determined by the application software, Analyst, version 1.5.2 (Applied Biosystems, Mississauga, Canada). The mass-to-charge transition (m/z) of precursor ions and product ions for each compound were identified as follows: m/z 152 → 110 for acetaminophen, m/z 156 → 114 for acetaminophen-D_4, m/z 256 → 139 for hydroxybupropion, m/z 262 → 139 for Hydroxybupropion-D_6, m/z 312 → 230 for 4'-hydroxydiclofenac, m/z 318 → 236 for 4'-Hydroxydiclofenac-$^{13}C_6$, m/z 342 → 203 for 1'-hydroxymidazolam and m/z 346 → 203 for 1'-Hydroxymidazolam-D_4. The concentrations were determined by the peak area ratio method using a calibration curve made up of 8 concentrations. In addition, three levels of QC samples (low, medium and high) were included in each analytical run to validate the results.

Cocktail 2 LC-MS/MS analytical method :

One volume of sample supernatant was mixed with two volumes of internal standard (IS) solution (containing 4'-hydroxymephenytoin-D_3, 5-hydroxyrosiglitazone-D_4 and dextrorphan-D_3), then evaporated to dryness under a gentle stream of nitrogen at 50°C and reconstituted in 1 volume of H_2O/ACN/formic acid (90/10/0.1, v/v/v). The UPLC system used was an Agilent 1290 series instrument (Agilent technologies Santa Clara, California, USA) coupled with a Sciex API 5000 mass spectrometer (Applied Biosystems, Mississauga, Canada). The analytical column was a Zorbax Eclipse plus XDB C18 (1.8 µm, 50 x 2.1 mm), operated at 30°C. Eluent A was water containing 0.1% formic acid and eluent B was methanol containing 0.1% formic acid. The flow-rate was 0.350 mL/min. A gradient elution was started at 95% A (1 min), ramped to 80% A in 1.5 min,

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held at 80% A for 0.5 min, then ramped to 20% A in 3 min and held at 10% A for 1 min before returning to the initial condition (95% A). Data acquisition was performed and the analytical parameters were determined by the application software, Analyst, version 1.5.2 (Applied Biosystems, Mississauga, Canada). The mass-to-charge transition (m/z) of precursor ions and product ions for each compound were identified as follows: m/z 235 → 150 for 4'-hydroxymephenytoin, m/z 238 → 150 for 4'-hydroxymephenytoin-D_3, m/z 258 → 157 for dextrorphan and m/z 261 → 157 for dextrorphan-D_3. The concentrations were determined by the peak area ratio method using a calibration curve made up of 8 concentrations. In addition, three levels of QC samples (low, medium and high) were included in each analytical run to validate the results.

LC-MS/MS analysis for monitoring parent drug disappearance

The disappearance (peak area) of CYP3A4 substrates used for the validation of the assay (felodipine, simvastatin, quinidine, sildenafil, nifedipine, cerivastatin, metoprolol, zolpidem, loperamide, mirtazapine and pimozide), as well as of the various phase II enzymes (1-hydroxymidazolam, 4-hydroxycoumarin and 5-hydroxytryptophol) and AO (phthalazine) were measured by the following UPLC-MS/MS method. Samples were diluted 4-fold with water before analysis. The UPLC system used was an Agilent 1290 series instrument (Agilent technologies Santa Clara, California, USA) coupled with a Sciex API 5000 mass spectrometer (Applied Biosystems, Mississauga, Canada). The analytical column was an Acquity UPLC HSS T3 (1.8µm, 2.1x30mm), operated at 40°C. Eluent A was water containing 0.1% formic acid and eluent B was ACN containing 0.1% formic acid. The flow-rate was 1 mL/min. A gradient elution was started at 95% A, ramped to 30% A in 1 min, held at 10% A for 0.3 min before return to the initial condition (95% A for 0.44 min). Analytical parameters were determined by the software, Discovery Quant Analyze,

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version 2.1.1 (Applied Biosystems, Mississauga, Canada). Data acquisition was performed by the application software, Analyst, version 1.5.2 (Applied Biosystems, Mississauga, Canada).

UPLC – HRMS analysis for monitoring specific metabolite formation

For cimetidine, ranitidine, CDP323, imipramine, propofol, naloxone and S-oxazepam, the metabolite formed by the enzyme of interest was monitored by UPLC-HRMS. Samples were diluted 2-fold with water before analysis. An Acquity UPLC instrument (Waters Corporation, Manchester, UK) coupled to a XevoG2S Qtof high resolution mass spectrometer (Waters, Manchester, UK) was used to perform the analysis. The analytical column was an Acquity UPLC HSS C18 (1.7µm, 2.1x100mm), operated at 40°C. Eluent A was water containing 10 mM ammonium actetate with 0.1% acetic acid and eluent B was ACN. The flow-rate was 0.400 mL/min. A gradient elution was started at 90% A (for 1 min), ramped to 10% A in 8 min, held at 10% A for 1.5 min before return to the initial condition (90% A for 1.49 min). Data acquisition was performed by the application softwares MassLynx, version 4.1, SCN884 and MetaboLynx, version 4.1, SCN871 (Waters Corporation, Mildford, Massachusetts, USA).

Data analysis

The CYP450 probe substrate metabolite formation rates were calculated using the following equation:

$$v = \frac{[metabolite]}{t * [hepatocytes]}$$

Where: [metabolite] is the concentration of P450 probe substrate metabolite produced during the incubation; t is the incubation time and [hepatocytes] is the cell density of hepatocytes in incubates. Percentage of inhibition caused by azamulin was calculated using zero-azamulin samples as control values.

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The *in vitro* intrinsic clearance determined from parent drug disappearance was calculated using the half-life ($t_{1/2}$) of compound disappearance using the following equation:

$$CL_{int} = -\frac{\lambda_z}{[hepatocytes]}$$

Where: λ_z was the slope obtained from linear regression of the natural logarithm of the compound peak area ratio in relation with time and [hepatocytes] was the cell density of hepatocytes expressed in million hepatocytes per mL.

For cimetidine, ranitidine, CDP323, imipramine, propofol, naloxone and S-oxazepam, no reference standards were available to quantify the metabolite formation rate. Therefore, the peak area of the metabolite of interest was used to measure the slope (peak area over time) as a marker of the formation rate. This slope was determined for both conditions (presence and absence of azamulin) and used to determine the effect of azamulin on the metabolic pathway of interest.

Fraction metabolized by CYP3A4/5 ($f_{m,CYP3A4/5}$) was calculated for each compound from the ratio between CL_{int} (based on parent drug disappearance) or metabolite formation rate [v] (or slope corresponding to metabolite formation rate) in absence and in presence of azamulin, by using the following equation :

$$Fm_{CYP3A4/5} = 1 - \frac{CL_{int\ with\ azamulin}}{CL_{int\ without\ azamulin}} = 1 - \frac{v_{with\ azamulin}}{v_{without\ azamulin}}$$

IC_{50} values were determined by non-linear regression analysis using 4-parameters equation in GraphPad Prism (version 7.02, GraphPad Software, Inc, La Jolla, California, USA) with top and bottom parameters set to 100 and 0, respectively.

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Statistical Analysis

The accuracy of the prediction was assessed and compared from root mean-squared error (RMSE) and average fold error (AFE) calculated with the following equations (Sheiner and Beal, 1981; Obach et al., 1997).

$$RMSE = \sqrt{\frac{1}{N}\sum(Predicted - Observed)^2}$$

$$AFE = 10^{\frac{1}{N}\Sigma\left[log\left(\frac{Predicted}{Observed}\right)\right]}$$

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RESULTS

Evaluation of the time-dependent inhibitory effect of azamulin on CYP3A4/5 activity, in suspensions of pooled human hepatocytes

Since azamulin was known to be a time-dependent inhibitor in HLM, we first evaluated the effect of the pre-incubation time on the IC_{50} of azamulin. As shown in Table 1 and Figure 1 (panel A), even without pre-incubation, azamulin was a potent CYP3A4/5 inhibitor with an IC_{50} value of 1.0µM. A pre-incubation step as short as 10 min already increased its potency by 2-fold (IC_{50} of 0.46µM). Longer pre-incubation steps did not significantly increase its inhibitory potency. Indeed, a 22-fold increase of the pre-incubation time (220 min) only led to an additional 3-fold increase in potency (0.13µM) compared to the 10 min pre-incubation. During these preliminary investigations, the metabolic stability of azamulin in human hepatocytes (over 4 h incubation) was also determined and showed that over 40% of azamulin remained present after 4 h (Figure 1, panel B). The major metabolic pathway of azamulin was found to be mediated by direct glucuronidation (data not shown).

Evaluation of the selectivity of azamulin towards CYP3A4/5, in suspensions of pooled human hepatocytes

The purpose of the present work was to set-up an assay in suspensions of pooled human hepatocytes to determine the $F_{mCYP3A4/5}$ of NCE's by measuring the effect of azamulin on parent drug disappearance, which is usually measured over a 4 h incubation time. It was therefore deemed critical to ensure CYP3A4/5 selectivity of azamulin over this 4 h incubation period. To check this, the inhibitory effect of azamulin on major P450 enzymes (CYP1A2, 2B6, 2C8, 2C9, 2C19 and 2D6) was investigated using formation of selective metabolite for each P450.However, to respect initial rate conditions for metabolite formation, the incubations with each probe substrate had to be performed within a timeframe of maximum 1 h. The 4 h incubation period could not be directly

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covered. Therefore, to overcome this limitation and allow the evaluation of the CYP3A4/5 selectivity of azamulin over 4 h period, a dedicated assay design was applied. . The inhibition potential of azamulin towards other CYP450 was determined using two different conditions: (i) without pre-incubation of azamulin (azamulin and selective probe susbtrate were co-incubated for maximum 1h) and (ii) with a 3-hour pre-incubation with azamulin (probe substrate is added at the end of 3 h pre-incubation for maximum 1 h incubation in presence of remaining azamulin). As shown in Figure 2 (panel A), azamulin (3 µM) caused less than 20% of inhibition of all CYP450 enzymes tested, with and without 3 h pre-incubation (for most of the CYP450s even less than 10%). These data clearly demonstrate that azamulin is very specific to CYP3A over a 4 h incubation period. In contrast, ketoconazole (3 µM – panel B) showed a lack of CYP3A4 selectivity as it significantly inhibited (>20% inhibition) CYP2C9, CYP2C19 and CYP2B6.

The main advantage of using a hepatocyte assay is the presence of a full array of drug-metabolizing enzymes (DME). It was therefore deemed critical to assess as much as possible the potential inhibition of azamulin towards non-CYP450 enzymes. To this aim, the inhibitory effect of azamulin on the metabolism of 1'hydroxymidazolam (UGT1A4, 2B4, 2B7) (Seo et al., 2010), hydroxycoumarin (UGTs and SULTs substrate), phthalazine (aldehyde oxidase substrate) (Barr and Jones, 2011), cimetidine and ranitidine (FMO substrate) (Cashman et al., 1993), CDP323 (CES1 substrate) (Chanteux et al., 2014) and several specific UGT substrates (imipramine for UGT1A4, 5-hydroxytryptophol for UGT1A6, S-oxazepam for UGT2B15, Propofol for UGT1A9, naloxone for UGT2B7) (Court et al., 2002; Krishnaswamy et al., 2004; Mukai et al., 2014) was also evaluated. The effect of azamulin was measured either using parent drug disappearance or specific metabolite formation for compounds known to have other major metabolic pathways not representative of the target enzyme. The percentages of inhibition caused by azamulin are presented in Table 2. Azamulin caused a very modest inhibition (20-30%) of some UGTs (1'-hydroxymidazolam,

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naloxone and oxazepam) and FMO (ranitidine) substrates but it had no significant effect (<15% inhibition) on the other metabolic pathways evaluated (glucuronidation and sulfation of hydroxycoumarin, glucuronidation of 5-hydroxytryptophol and propofol, phthalazine oxidation by aldehyde oxidase, cimetidine oxidation by FMO). Additional data with recombinant UGT enzymes expressed in the liver (UGT1A1, 1A3, 1A4, 1A9, 2B4, 2B7, 2B10, 2B15, 2B17) were generated to complement the findings observed with 1'-hydroxymidazolam and S-oxazepam in suspended human hepatocytes. Though UGT2B15 was confirmed to be the primary UGT isoform involed in S-oxazepam glucuronidation, UGT1A9, UGT2B4 and UGT2B7 were also found to contribute to a lesser extent to the formation of S-oxazepam glucuronide. Regarding glucuronidation of 1'-hydroxymidazolam, only UGT1A4, 2B4 and 2B7 were identified as UGT isoforms involved in this reaction. In this experimental setting (recombinant UGT incubated in presence or absence of azamilin at 3µM), azamulin also demonstrated weak inhibition potential of UGT1A9 (24% with S-oxazepam), UGT2B4 (23% with S-oxazepam and 30% with 1'-hydroxymidazolam), UGT2B7 (23% with 1'-hydroxymidazolam) and UGT2B15 (35% with S-oxazepam). No significant inhibition (<10%) was observed in UGT1A4 (1'-hydroxymidazolam) and UGT2B7 (S-oxazepam) (data not shown).

Evaluation of the inhibition of midazolam 1'-hydroxylation, in CYP3A5-genotyped human hepatocytes : comparison with CYP3cide

In the work of Stresser et al, azamulin demonstrated a 10-fold more potent inhibition against CYP3A4 versus CYP3A5 using recombinant enzymes and 7-benzyloxy-4-trifluoromethylcoumarin as probe substrate for both CYP enzymes. More recently, Parmentier et al (Parmentier et al., 2017) demonstrated that azamulin specifically inhibited CYP3A4 and not CYP3A5 in HLM. In the present study, we have also looked at the CYP3A selectivity of azamulin. To this aim, , we used an indirect approach to determine whether azamulin, in the conditions of our assay (incubation at 3µM and

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midazolam as CYP3A4/5 probe substrate), would still demonstrate an apparent selectivity for CYP3A4. To achieve this, the effect of azamulin on the 1'-hydroxylation of midazolam was investigated in CYP3A5-genotyped human hepatocytes. Incubations were performed in parallel with CYP3cide, which is a well-known selective CYP3A4 inhibitor (Walsky et al., 2012). The inhibition obtained in presence of azamulin and CYP3cide are shown in Figure 3. In both batches of hepatocytes with the CYP3A5*3/*3 genotype (non-expressers of CYP3A5, panel A), a high inhibition (~ 90%) was observed, as expected, with both CYP3cide and azamulin. On the other hand, in batches of human hepatocytes with the CYP3A5*1/*1 genotype (high CYP3A5 expressers, panel B), the 1'-hydroxylation of midazolam was still significantly inhibited (~ 85%) by azamulin while, the inhibition observed in presence of CYP3cide was significantly lower (29% and 61%). Overall, these data demonstrated that azamulin (3µM) is not as selective as CYP3cide for CYP3A4 as it also inhibits the CYP3A5 isoform.

Validation of the phenotyping assay with a set of well-known CYP3A4/5 substrates

In order to validate the use of this assay as a new tool to determine $F_{m,CYP3A4/5}$ of NCE's, the inhibitory effect of azamulin (3 µM) on intrinsic clearance of a set of 11 compounds, which are reported to be highly, moderately, poorly or not metabolized by CYP3A4/5 (midazolam, felodipine, simvastatin > quinidine, sildenafil, nifedipine, loperamide > zolpidem, pimozide, cerivastatin, mirtazapine > metoprolol), was evaluated in suspended human hepatocytes according to the experimental conditions described in Table 3. The CL_{int} values obtained for each compound in absence and in presence of azamulin are presented in Table 4 together with the predicted and observed $F_{m,CYP3A4/5}$. In addition, the graphs showing the disappearance of each compound in presence and in absence of azamulin are also presented in Figure 4. As illustrated in Figure 5, the correlation between the predicted and observed $F_{m,CYP3A4/5}$ is pretty good ($r^2 = 0.77$). In addition, the AFE (0.97) is close to 1 and the RMSE is only 0.12 supporting that the experimental conditions

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used in the present study are adequate for determining the in vivo CYP3A contribution in drug's metabolic clearance. Prediction of $F_{m,CYP}$ is quite challenging, especially when the $F_{m,CYP}$ is in the range of 0.3-0.7 where a huge interindividual variability is observed due to the presence of multiple pathways all of which may vary among individuals (Desbans et al., 2014). Therefore, it is not surprising to see that the 4 compounds (loperamide, nifedipine, pimozide and zolpidem) being slightly outside the 10% boundaries have an observed $F_{m,CYP3A4/5}$ in this range (0.3-0.7) where high inter-individual variability occurred. It should not be forgotten that in vitro determination of $F_{m,CYP3A4/5}$, is highly dependent on the intrinsic CYP3A4/5 activity in the batch of hepatocytes used. Therefore, the selection of the human hepatocytes batch is primordial to ensure an appropriate prediction of the in vivo $F_{mCYP3A4/5}$.

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DISCUSSION

Nowadays it is well-recognized that an early evaluation of the contribution of individual P450 isoforms to the total metabolic clearance of a NCE is very important in order to predict the risk of victim DDI, which could occur by co-administration with other drugs affecting P450 activity. This can lead to a loss or increase of the pharmacodynamic effects and/or serious side effects. In addition to DDI risk, knowledge of DME in clearance of NCE is primordial to inform on risks associated with modulation of DME in certain disease states (Harvey and Morgan, 2014), in specific populations (pediatrics) or due to inherent variability associated with abundance or the polymorphic nature of specific DME (e.g. CYP2D6, UGT1A1). DDIs not only affect patient safety but also add to the cost of drug development because of the high costs of failure in clinical development (Xu et al., 2009). Consequently, identifying the risks associated with DDI play an increasingly important role in the pharmaceutical industry, contributing to the selection of suitable candidates for (pre)clinical development and to support clinical pharmacologists to establish a suitable clinical DDI trial design (Desbans et al., 2014).

It is therefore critical to understand early in drug development the contribution of individual CYP450 enzymes to metabolism of NCE (fractional CL_{int} - $F_{CL,metabolism}xF_{m,CYP}$) in order to anticipate and mitigate the risk of DDI as victim, since DDI clinical trials are usually conducted in later stages of clinical development (post proof-of-concept). Even though the RAF/ISEF approach is widely used to determine the fractional CL_{int} (Bohnert et al., 2016), this method suffers from one major limitation. Indeed, it is assumed that drug candidates interact with the P450 binding site in the same manner as the probe substrate used for RAF/ISEF determination and, therefore, that RAF/ISEF are similar between substrates. This main assumption may not be valid knowing the presence of multiple binding sites with different substrate selectivities described for several P450 isoforms (Venkatakrishnan et al., 2000; Crewe et al., 2011; Siu and Lai, 2017). Therefore, the use of

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a specific inhibitor is still an interesting alternative. However, for this approach to be successful, the inhibitor should be potent, selective, metabolically stable to be able to attain >90% inhibition of the targeted enzyme over the incubation period. Based on the limited literature data available so far (Stresser et al., 2004; Parmentier et al., 2017), the potent CYP3A inhibitor azamulin, has potentially better properties to meet these criteria compared to the gold standard CYP3A inhibitor, ketoconazole, which mainly suffers from a lack of selectivity (Liu et al., 2007), high non-specific binding (Tran et al., 2002; Quinney et al., 2013) and metabolic instability (Stresser et al., 2004). Therefore, in the present study, we aimed to fully characterize azamulin as a potential tool for the estimation of CYP3A contribution to drug metabolism. Human hepatocytes were selected as test system in order to directly quantify $F_{CL,metabolism}xF_{m,CYP3A}$ since they contain the whole enzymatic inventory involved in drug metabolism. The first objective of the study was to confirm the inhibitory properties of azamulin in human hepatocytes. As demonstrated previously in human liver microsomes (Stresser et al., 2004; Parmentier et al., 2017), azamulin also showed in human hepatocytes a time-dependent inhibition profile as its IC_{50} decreased with an increase of the pre-incubation time. An optimal concentration (3 µM) was selected to achieve >90% CYP3A inhibition for incubations lasting up to 4h.

Secondly, azamulin was tested for its CYP3A4/5 selectivity towards other CYP450 enzymes and a wide panel of DMEs. The present data clearly demonstrated the superiority of azamulin compared to ketoconazole regarding P450 selectivity. Indeed, less than 20% inhibition of CYP1A2, 2B6, 2C8, 2C9, 2C19, 2D6 was observed in presence of 3 µM azamulin while ketoconazole significantly inhibited CYP2B6 and CYP2C19 at concentrations required to achieve >90% CYP3A4/5 inhibition. These findings obtained in human hepatocytes are similar to those described by Stresser et al advocating the higher selectivity of azamulin over ketoconazole in human liver microsomes and recombinant enzymes (Stresser et al., 2004).

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These encouraging data triggered additional investigations on the potential impact of azamulin on other drug metabolizing enzymes (FMO, carboxylesterase, UGTs, SULTs, AO). This was deemed very important to assess since human hepatocytes were used as test system. Again, azamulin demonstrated no or minimal inhibition (<15%) of these enzymes with the exception of some UGTs like UGT2B7, UGT2B4 and UGT2B15 which demonstrated modest inhibition (~20-30%). This is most likely due to competitive inhibition as glucuronidation was found to be the main metabolic pathway of azamulin. It should be noted that some key hepatic UGTs like UGT1A1 and UGT1A3 were not evaluated in the current work. The present data showed a trend of weak inhibition on some UGTs which needs to be considered in the interpretation of the data when direct glucuronidation of parent drug is expected or known. However, the impact on the CYP3A determination is unlikely to be significant owing to the minor inhibition observed.. . Overall, these data support the use of azamulin as a selective CYP3A inhibitor in human hepatocytes without impacting significantly other major DME.

Finally, we have assessed the selectivity of azamulin for CYP3A4 over CYP3A5. Indeed, understanding the relative contribution of CYP3A4 and CYP3A5 to drug metabolism is important primarily because expression of the CYP3A5 enzyme is subject to a genetic polymorphism wherein most humans do not express it (Lamba et al., 2002) and some CYP3A substrates showed different disposition profiles in extensive and poor CYP3A5 metabolizers (Haufroid et al., 2006; Jin et al., 2007). In the absence of specific CYP3A4 and CYP3A5 probe substrates, we used genotyped human hepatocytes (non-CYP3A5 expressers and high CYP3A5 expressers) together with CYP3cide, a well-known specific CYP3A4 inhibitor (Tseng et al., 2014) to characterize the CYP3A4/CYP3A5 selectivity of azamulin in our assay conditions. Our data suggest that azamulin, under our conditions (3µM in human hepatocytes) is a CYP3A4/5 inhibitor and cannot be used to determine the relative CYP3A4/5 contribution. While CYP3cide is a useful inhibitor for that

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purpose, azamulin is more appropriate for DDI prediction with CYP3A inhibitors as most CYP3A4 inhibitors also inhibit CYP3A5 enzymes.

Our observations on the lack of CYP3A4/5 selectivity of azamulin are in contradiction to the conclusions drawn recently by Parmentier and coworkers. Though test systems (recombinant enzymes and HLM) were different, azamulin concentration (5 µM) was in the same range (Parmentier et al., 2017). In their experiment with CYP3A4-silensomes (HLM prepared with pre-treatment with azamulin), they showed that an additional reduction of midazolam and nifedipine clearance (15-20%) was observed in the CYP3A4-silensomes in presence of ketoconazole (0.3µM, pan-CYP3A inhibitor). Authors have ascribed the remaining CYP3A activities to CYP3A5 only. Even though this interpretation may be a plausible explanation of the data, it should have been supported by stronger evidences. Indeed, it cannot be excluded that the additional effect of ketoconazole is due to remaining CYP3A4 activities since full CYP3A4 inhibition in CYP3A4-silensomes has not been demonstrated. In addition, we cannot rule out that a significant proportion of CYP3A5 has been already inhibited during preparation of CYP3A4-silensomes since no information on the CYP3A5 genotype and/or activity present in their HLM batch has been provided. Finally, the evidence of an additional effect of ketoconazole were based on sub-optimal experimental conditions. Indeed, the effect of ketoconazole was determined in conditions where metabolic clearance of midazolam and nifedipine were already very low (more than 95% parent drug remaining at the end of the assay in the control conditions). Under such conditions (very low turn-over of parent drug), any intrinsic clearance determination based on parent drug consumption is expected to be inaccurate and should not be used to determine a potential additional effect of ketoconazole.

Stresser et al (Stresser et al., 2004) suggested that azamulin was 10-fold more selective for CYP3A4 over CYP3A5 using recombinant CYP3A4 and CYP3A5 enzymes. However, this has been

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assessed in a competitive inhibition model with 7-benzyloxy-4-trifluoromethylcoumarin as probe substrate. Results obtained with recombinant CYP450 always need to be handled with caution as they are difficult to extrapolate. Differences in the expression levels of NADPH-P450 reductase and cytochrome b5 (Emoto and Iwasaki, 2007) or differences in activity due to different lipidic environment, pH or oxidative stress may have an impact on the translation of data from recombinant P450 to liver microsomes or hepatocytes (Marheineke et al., 1998; Brignac-Huber et al., 2016). Also, a 10-fold difference is clearly not enough to ensure full inhibition of CYP3A4 (>90%) on one hand and no or very little inhibition of CYP3A5 on the other hand.

In summary, azamulin (3 µM) is a highly potent and selective inhibitor of CYP3A in human hepatocytes. The assay presented in the current study can be used to accurately assess the in vivo contribution of CYP3A in the metabolic clearance of any NCE and inform on the risk of DDI with CYP3A inhibitors. However, it should be acknowledged that one clear limitation of the current assay is the determination of the $F_{mCYP3A4/5}$ when NCE have very low turn-over in human hepatocytes. Further work is required to validate the use of relative metabolite formation (in absence of authentic reference standards) to determine the CYP3A4/5 contribution to NCE metabolic clearance.

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AUTORSHIP CONTRIBUTIONS

Participated in research design: HC, MR, JN, AL

Conducted experiments: MR, JN, EG, CD, SD

Contributed new reagents or analytic tools: EG, CD, SD

Performed data analysis: HC, MR, CD, JN, AL

Wrote or contributed to the writing of the manuscript: HC, MR

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FOOTNOTES

Not applicable

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LEGENDS FOR FIGURES

FIGURE 1

(A) IC_{50} curves for the effect of azamulin on the formation of 1'-hydroxymidazolam from Midazolam (5µM) in suspended human hepatocytes (0.5 x 10^6 cells/mL) at different pre-incubation times (0 min (•), 10 min (Δ), 100 min (∇) and 220 min (◊)). Points represent mean values ± SD (n=3). IC_{50} values are shown in Table 1.

(B) Stability of azamulin (3µM) in suspended human hepatocytes (1 x10^6 cells/mL). Points represent singular values of 3 independent experiments.

FIGURE 2

Inhibitory effect of Azamulin (3µM) (A) and ketoconazole (3µM) (B) on major CYP450 enzymes (CYP1A2, 2B6, 2C8, 2C9, 2C19, 2D6 and 3A4) in suspended human hepatocytes (1 x 10^6 cells/mL) after 0 min (closed bars) or 3 h (open bars, azamulin only) pre-incubation in the presence of inhibitor. Bars represent mean values ± SD (n=3).

FIGURE 3

Comparison of midazolam 1'-hydroxylation inhibition by azamulin (open bars) and CYP3cide (closed bars), in CYP3A5 genotyped hepatocytes. Two different genotypes were tested: CYP3A5*3/*3 (panel A, batch JYS and UEK, non expressers of CYP3A5) and CYP3A5*1/*1 (panel B, batch RSA and QWG, high expressers of CYP3A5). Bars represent mean values ± SD (n=3).

FIGURE 4

Metabolic disappearance of CYP3A4/5 substrates with various $f_{m,CYP3A4/5}$ in suspended human hepatocytes (1x10^6 cells/mL) in absence (○) or presence (●) of azamulin (3 µM). Results of one single experiment are shown and are representative of 3 independent experiments.

FIGURE 5

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Correlation between observed in vivo and predicted $F_{m,CYP3A4/5}$. All $F_{m,CYP3A4/5}$ values are shown in Table 4. The solid line represents the line of unity and the dotted line the ± 10% error interval. Points represent $F_{m,CYP3A4/5}$ calculated from 3 independent experiments.

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TABLES

TABLE 1 : Time-dependency of the inhibitory effect of azamulin on CYP3A4/5

Pre-incubation time (min)	IC_{50} ± SE (µM)
0	1.0 ± 0.1
10	0.46 ± 0.02
100	0.20 ± 0.02
220	0.13 ± 0.01

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TABLE 2 : Evaluation of the selectivity of azamulin towards CYP3A4 in suspended human hepatocytes : determination of its inhibitory effect on phase II enzymes, aldehyde oxidase, FMO and CES1

Substrate	Enzyme	Reaction monitored	Percentage of inhibition*
1'-Hydroxymidazolam	UGT1A4, 2B4, 2B7	parent disappearance	26 (14)
4'-Hydroxycoumarin	UGTs, SULTs	parent disappearance	4 (8)
Phthalazine	Aldehyde oxidase	parent disappearance	-0.6 (6)
Cimetidine	FMO	metabolite formation	13 (13)
Ranitidine	FMO	metabolite formation	24 (4)
CDP323	CES1	metabolite formation	-7 (23)
Imipramine	UGT1A4	metabolite formation	-6 (13)
5-Hydroxytryptophol	UGT1A6	parent disappearance	-2 (13)
Propofol	UGT1A9	metabolite formation	-3 (0.1)
Naloxone	UGT2B7	metabolite formation	29 (5.4)
S-Oxazepam	UGT2B15	metabolite formation	31 (8.4)

* Mean of 3 independent values (SEM)

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Table 3 : Incubation Conditions for Routine Assessment of $F_{mCYP3A4/5}$ of NCE

Culture plate	48-well
Culture medium	William's medium E without phenol red, containing 2 mM of glutamine and 15 mM of Hepes
Incubation	37°C in 5% CO_2/95% air, humidified atmosphere under agitation (450 rpm)
Hepatocytes suspension pre-warming	30 min at 2 millions/mL in similar conditions as incubation
Final incubation volume	500 µL
Final hepatocyte concentration	1 millions/mL
Substrate concentration	0.5 µM
Azamulin concentration	3 µM
Final organic solvent concentration	1% ACN
Incubation time	4 h
Sampling time	0, 30, 60, 120, 180 and 240 min
Sampling volume	50 µL/timepoint

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Table 4 Determination of CYP3A4/5 contribution to the metabolic clearance obtained in human hepatocytes for 12 marketed drugs

Compounds	CL_{int} (µL/min/10^6 hep)		Predicted in vitro $F_{m,CYP3A4/5}$	Observed in vivo $F_{m,CYP3A4/5}$	References
	Control	With azamulin			
Midazolam	56±4.6	3.4±0.6	0.94±0.01	0.84-0.99	(Galetin et al., 2006; Shou et al., 2008; Xu et al., 2011)
Felodipine	69±5.4	9.9±1.0	0.86±0.01	0.81-0.99	(Galetin et al., 2006)
Simvastatin	153±16	27±1.0	0.82±0.01	0.83-0.99	(Shou et al., 2008; Xu et al., 2011)
Quinidine	2.9±0.5	0.81±0.3	0.73±0.04	0.61-0.76	(Galetin et al., 2006; Shou et al., 2008)
Sildenafil	29±2.4	8.3±1.3	0.71±0.02	0.66-0.82	(Shou et al., 2008; Xu et al., 2009)
Nifedipine	56±5.5	10±1.7	0.82±0.02	0.63-0.71	(Galetin et al., 2006; Shou et al., 2008; Xu et al., 2011)
Loperamide	15±0.4	7.6±0.7	0.48±0.06	0.62	(Tayrouz et al., 2001)
Zolpidem	2.7±0.5	0.97±0.3	0.63±0.11	0.41	(Xu et al., 2011)
Pimozide	7.6±0.7	2.7±0.5	0.65±0.07	0.40	(Galetin et al., 2006)
Cerivastatin	7.2±0.7	5.8±3.4	0.46±0.03	0.37	(Galetin et al., 2006)
Mirtazapine	5.6±0.2	4.2±0.2	0.26±0.03	0.33	Notice FDA
Metoprolol	2.1±0.6	1.8±0.4	0.10±0.13	0.15	(Xu et al., 2009)

Date are Mean±SD of 3 independent experiments

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FIGURES

FIGURE 1

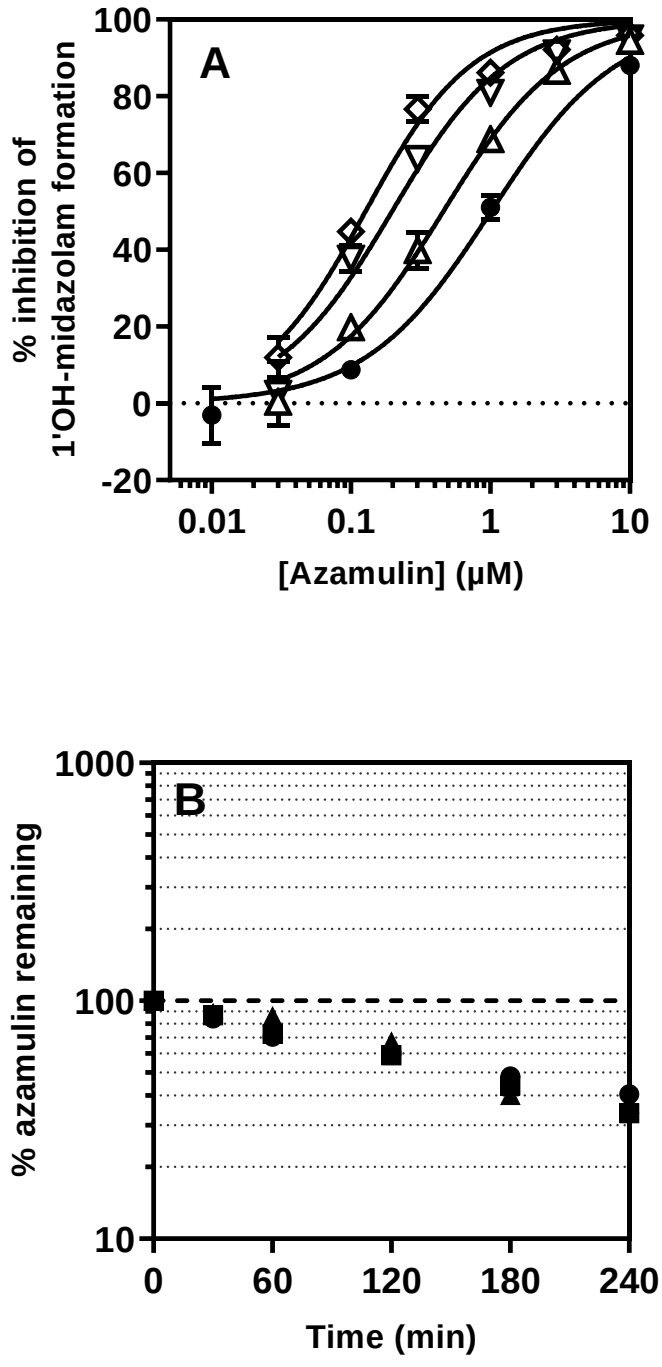

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FIGURE 2

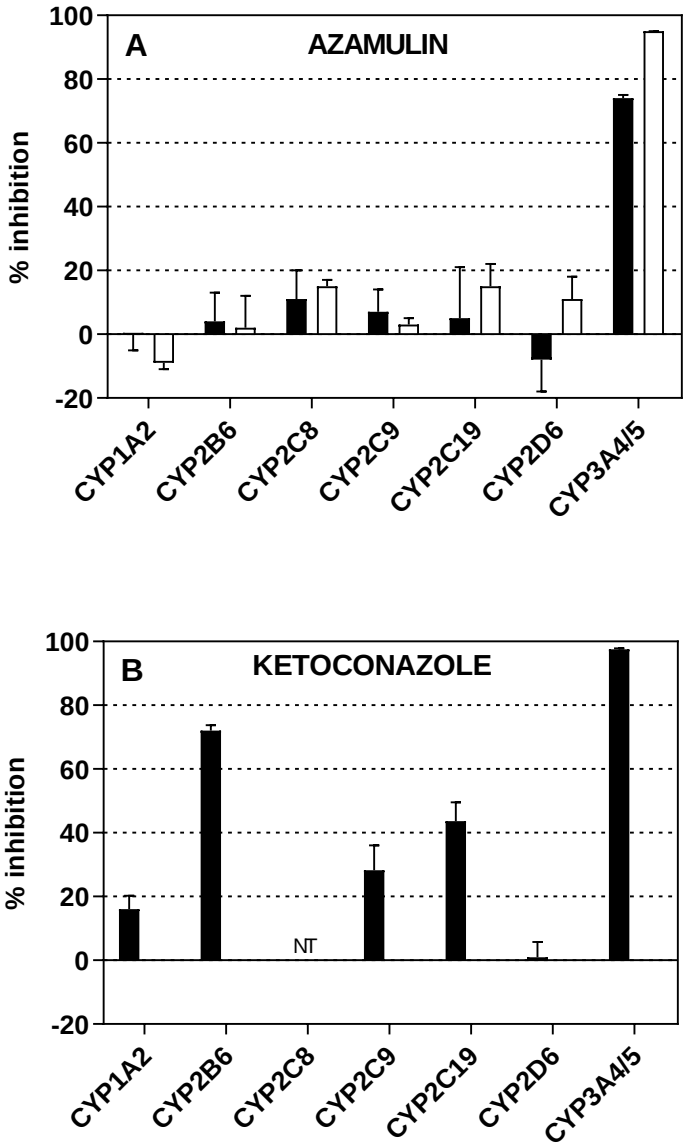

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Figure 3

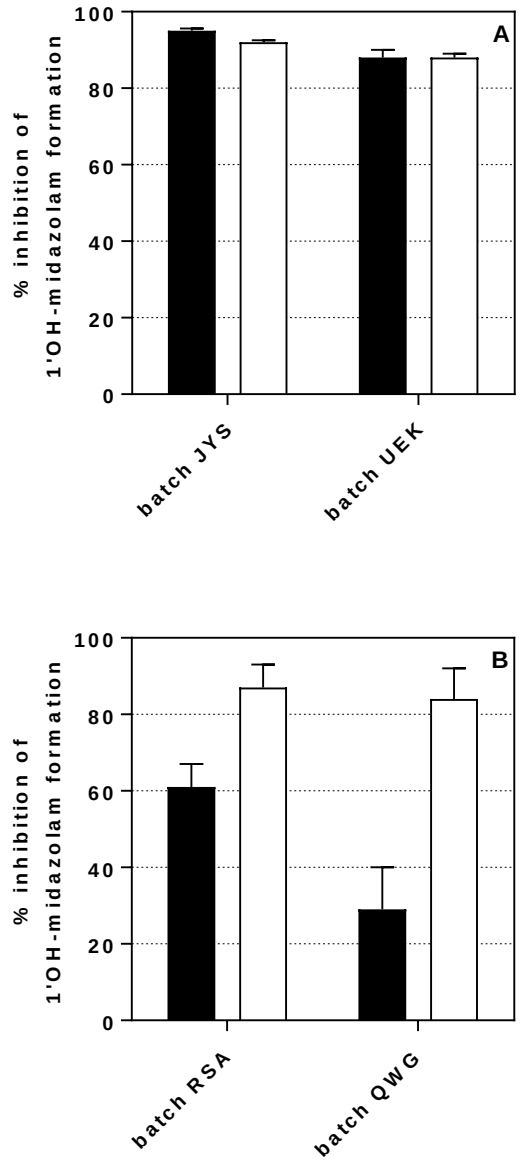

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FIGURE 4

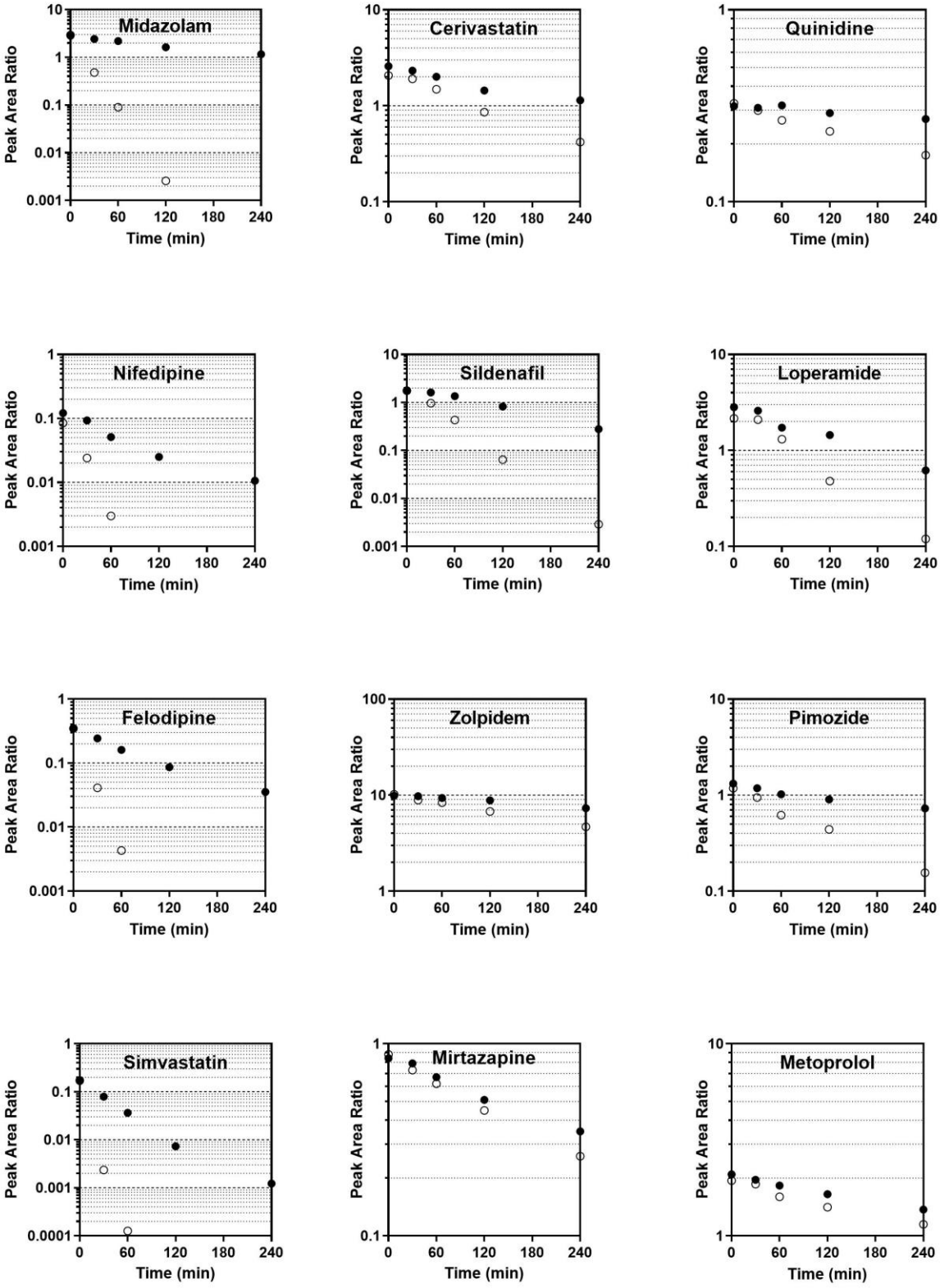

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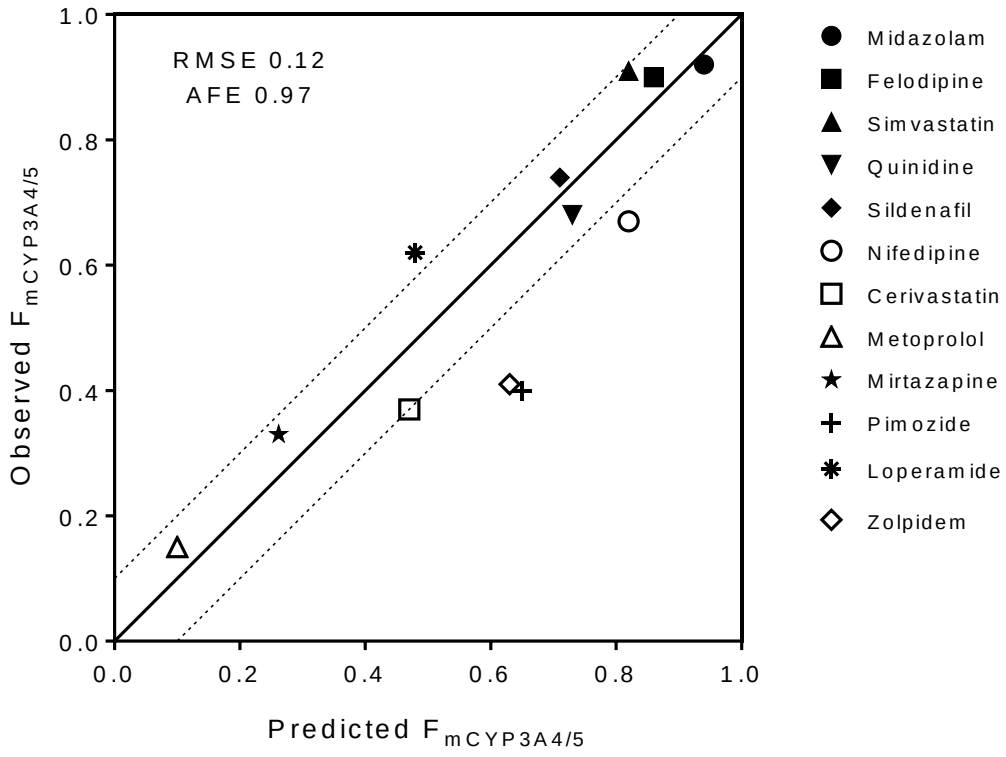
FIGURE 5



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